LYSOZYME IN EPIPHYSEAL CARTILAGE

II. The Effect of Egg White Lysozyme on Mouse Embryonic Femurs in Organ Cultures

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ABSTRACT

Embryonic mouse femoral cartilage, like the epiphyseal cartilage of the calf scapula, contains large amounts of lysozyme. The addition of egg white lysozyme to organ cultures of embryonic mouse femurs induces unique alterations in the gross and microscopic morphology of the femurs. The sites of these alterations are precisely related to the natural distribution of lysozyme in calf scapula. If the exogenous lysozyme is withdrawn from the culture, the morphological changes disappear, accompanied by a resumption or derepression of growth. The effect on growth is evident only in 17-day embryos. These observations support the idea that lysozyme has a physiological role in cartilage, perhaps related to a regulatory mechanism in bone formation.

It has now been over four decades since Sir Alexander Fleming discovered the bacteriolytic enzyme which he called lysozyme (1). Since that time there has been a great deal of investigation concerning its structure, precise mode of enzymatic activity, immunological properties, and distribution in nature (2–5). Although there appears to be some variability in the composition of lysozyme from different species and from different organs within the same species, these differences are relatively minor (6). All lysozymes are muramidases which split the β-1-4 linkage between muramic acid and *N*-acetylglucosamine (7). They also cleave the di- and tetrasaccharides of chitobiose and chitin, polymers of β-1-4 *N*-acetylglucosamines (8). Neither of these substrates is known to exist in mammalian tissues. The enzyme is a protein with a molecular weight of about 15,000 and a very basic isoelectric point. It is present in monocytic granules and is considered in these cells, and in some others, to be a lysosomal enzyme (9).

It is not generally known that, in his original studies of the distribution of lysozyme, Fleming found its concentration to be higher in cartilage than in any other tissue (1). More recently, our own observations have shown that in mammalian scapular cartilage the concentration of lysozyme is three times higher in the epiphyseal zones of columnar cells than elsewhere. Its concentration is higher in preosseous cartilage than in nonossifying cartilage, such as ear and nasal septum, and is higher in scapular cartilage from young dogs than in older ones (10). There is as yet no known substrate for lysozyme in cartilage. Experiments in which lysozyme was added to organ cultures of epiphyseal tissue from puppy scapulae have shown that this protein induced striking changes in gross appearance, characterized by an opacification of the explant which spared the area closest to the cartilage-bone junction. This

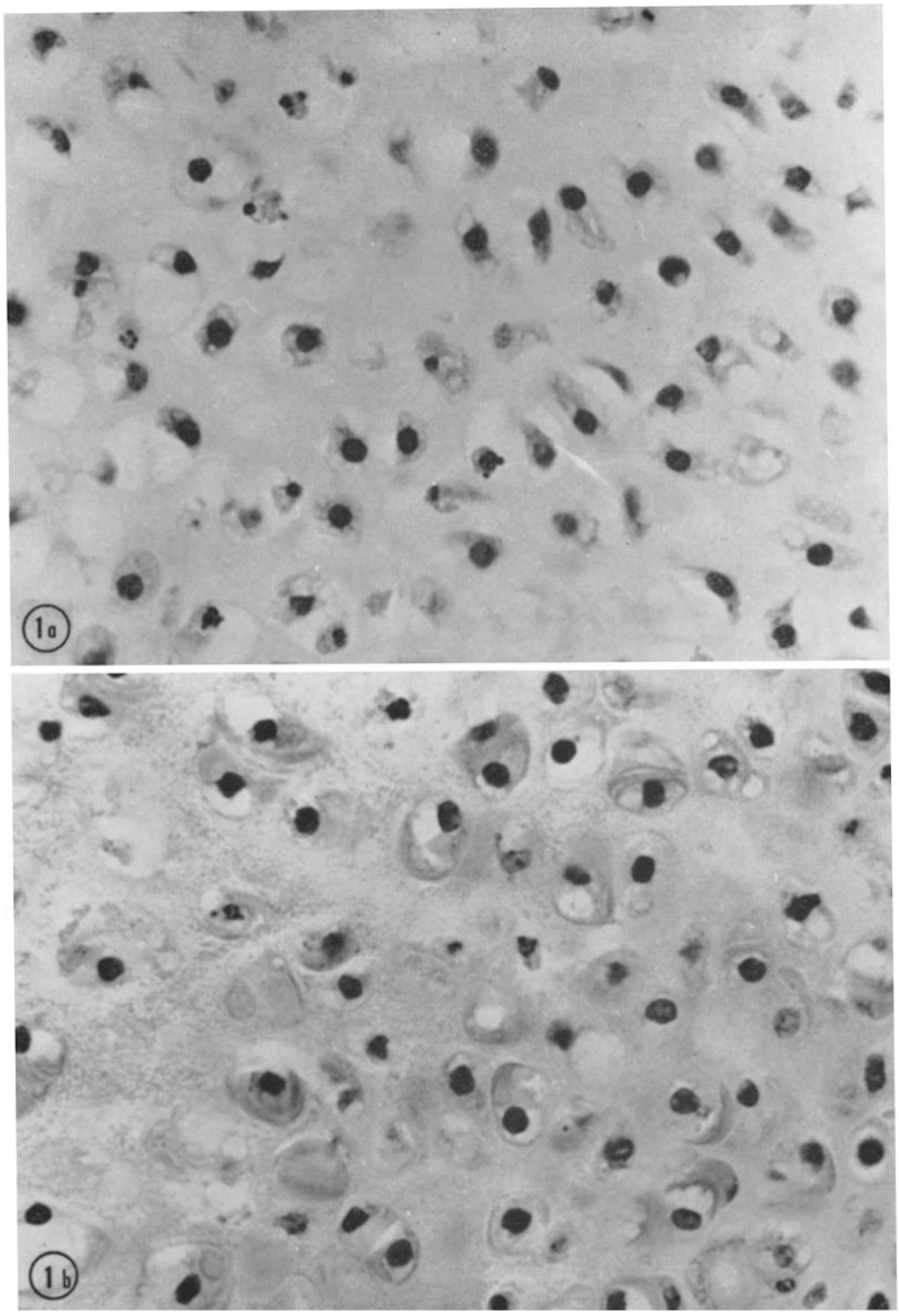
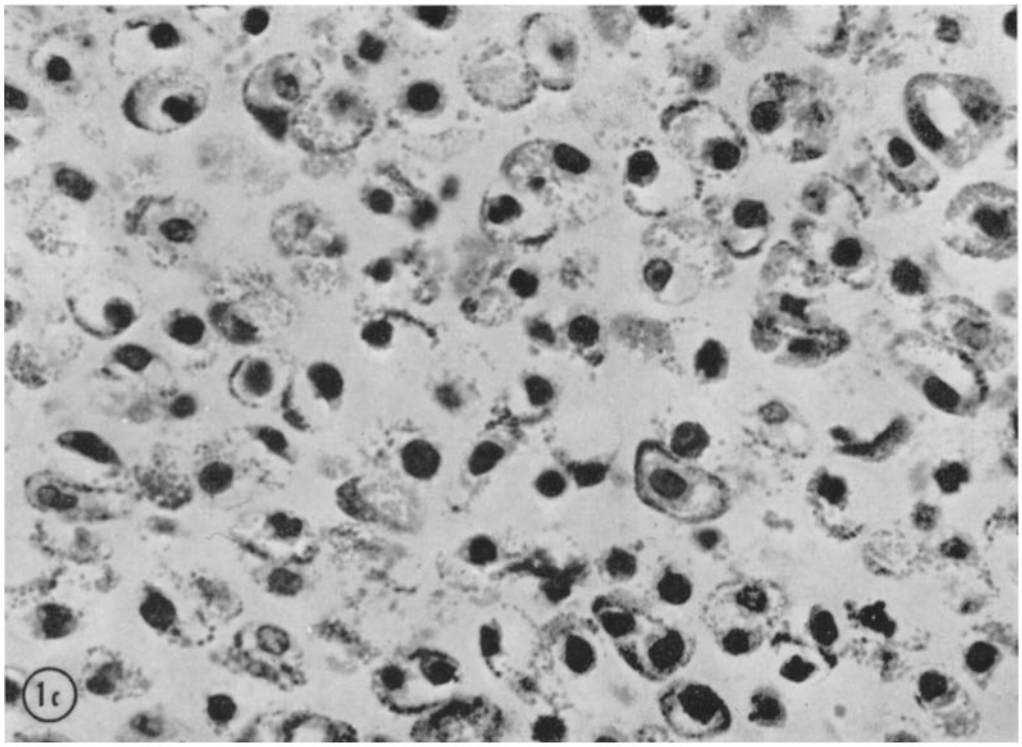


FIGURE 1 *a*, 17-day mouse femur, 7 days in lysozyme-free medium, resting cartilage. Hematoxylin and eosin stain. Approximately × 400. *b*, Similar specimen maintained for 7 days in medium containing 1 mg of lysozyme/ml. Eosinophilic deposits form dense crescentric capsules about chondrocytes and stippled droplets in matrix more distant from cells. Hematoxylin and eosin. Approximately × 400. *c*, Similar specimen maintained for 7 days in medium containing 1 mg of UV-inactivated lysozyme/ml. Droplets of eosinophilic material are confined to chondrocyte cytoplasm. Gomori's trichrome stain. Approximately × 400.



is precisely the zone in which lysozyme is normally present in highest concentration. The gross changes were accompanied by parallel alterations in the staining properties of the different regions of the cartilage matrix (11).

So as to confirm and extend these findings, it seemed of interest to determine what effects the addition of lysozyme might have on organ cultures of more rapidly proliferating and differentiating cartilage. Accordingly, experiments were designed for testing the effect of lysozyme on mouse embryonic femurs in organ culture.

METHODS AND MATERIALS

16- to 18-day-old embryonic mouse femurs from inbred mice of the Strong A strain were used in all experiments. The age of the embryos was calculated from the day of fertilization, as determined by the appearance of a vaginal plug. The embryos were obtained by Caesarean section. Embryonic age was confirmed by determination of the degree of ossification of the metatarsals of the hind foot as described by Wirtschafter (12). The femurs were aseptically dissected free of soft tissue in several baths of organ culture medium.

Individual femurs were cultured by means of the stationary plasma clot technique, as previously described (11). The explants were photographed periodically (at days 0, 3, 7, 10, and 14) during the course of the experiment. Experiments were terminated (usually 14 days after explantation) by fixing the tissue in 10% neutral buffered formalin. Histological studies were done on sections of paraffin-embedded tissues. In addition to untreated controls, experimental treatments included incubating explants in media containing egg white lysozyme (1 mg/ml of organ culture media), or equal concentrations of either bovine serum albumin, lysozyme inactivated by ultraviolet radiation, or chondromucoprotein (13). In some experiments, femurs were cultured for 7 days in the presence of 1 mg of lysozyme/ml and then cultured in identical medium free of lysozyme. In other experiments, the femurs were killed either by adding 10^{-4} M NaCN to the culture medium or by freezing to $-70°$ in dry ice and alcohol and then thawing at 37° five times. These dead femurs were maintained with and without lysozyme and compared to viable specimens cultured in identical media.

The egg white lysozyme[1] and bovine serum albumin were obtained commercially. Inactive lyso-

[1] Worthington Biochemical Corp., Freehold, New Jersey, egg white lysozyme, salt free, twice crystallized, code number 3.2.1.17.

zyme was obtained by irradiating a 0.2% solution of lysozyme in 10^{-3} N HCl for 7 hr with a high pressure quartz mercury vapor lamp. The irradiated lysozyme was incapable of lysing *Micrococcus lysodeikticus*, had an eltered absorption spectrum (14), but retained in an alectrophoretic field the mobility of a basic protein. At least 12 embryonic femurs were cultured for each treatment.

The relative rate of growth of the explant was calculated from planimetric measurements of the surface areas of identically enlarged photographs of the living explants in culture.

The amount of lysozyme in the tissue was assayed semiquantitatively by a modification of Schumacher's technique (15). This was done by inserting the specimen into a Petri dish containing a suspension of *Micrococcus lysodeikticus* in hypotonic agar, allowing it to gel and incubating in a wet chamber for approximately 40 hr at 37°C. The width of the zone of clearance in the agar surrounding the femur was proportional to the amount of diffusible lysozyme in the specimen. The results were recorded photographically.

RESULTS

Morphological Effects

When examined with transmitted light, the cartilaginous portions of femoral explants cultured in the presence of lysozyme appeared grossly more opaque than the control specimens. This change was visible within 36 hr after explantation and persisted for the duration of the experiment. As noted with puppy scapulae, this darkening spared the zone of the epiphysis closest to the cartilage-bone junction. In hematoxylin- and eosin–stained histological sections (Figs. 1 *a* and *b*), the bulk of the cartilage matrix in the resting zone was more eosinophilic in specimens cultured in the presence of lysozyme than in controls. This change was most marked in and about the chondrocyte lacunae. In the epiphyseal zones of columnar cells, the eosinophilic material formed a red-staining capsule around the chondrocytes; but the intercolumnar cartilage matrix stained like that of control specimens except for scattered fine droplets of eosinophilic material. These eosinophilic areas stained red with the periodic acid–Schiff (PAS) reagent even after diastase digestion, were not metachromatic with toluidine blue, and were red after coloration with Gomori's trichrome stain. Alterations of the staining properties of the matrix were most striking with the trichrome stain. The chrondrocytes also appeared to be somewhat larger than in control specimens. Neither cellular nor stromal changes were found in the focal areas of necrosis occasionally found in the specimens, nor did they occur in any of the various media when lysozyme was added to femurs killed with NaCN, or by freezing and thawing, or when the lower dose of lysozyme (0.1 mg/ml of medium) was used.

Explants cultured in media to which ultraviolet-irradiated lysozyme was added had different morphology (Fig. 1 *c*). Eosinophilic deposits with staining properties identical with those seen when native lysozyme was added were present, but they were restricted to the chondrocyte cytoplasm and were present as punctate droplets. Such droplets were not obvious in preparations exposed to active lysozyme.

In explants cultured in the presence of 1 mg of lysozyme/ml for 7 days and then maintained for an additional 7 days in lysozyme-free medium, the histological changes disappeared so that by the 14th day of culture (the time chosen for histological study) the explants were indistinguishable from femurs that were not exposed to lysozyme. The opacification of lysozyme-treated

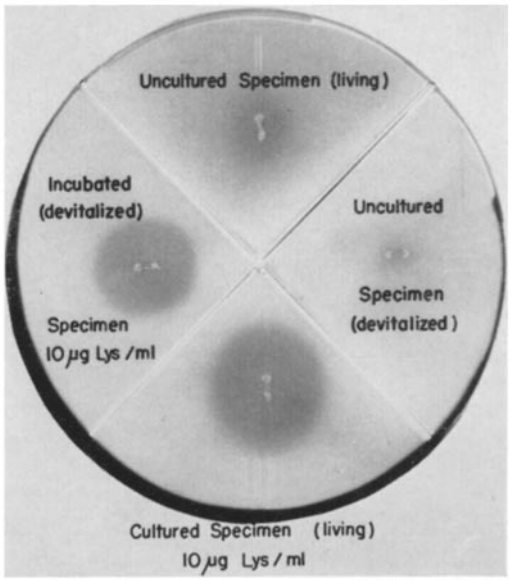


FIGURE 2 Lysozyme binding of living and devitalized embryonic mouse femur (17 days). Zones of lysis of *Micrococcus lysodeikticus* are larger in living than dead specimens. Both bind lysozyme from culture medium, but the width of the zone of lysis shows that living specimens bind more than dead ones.

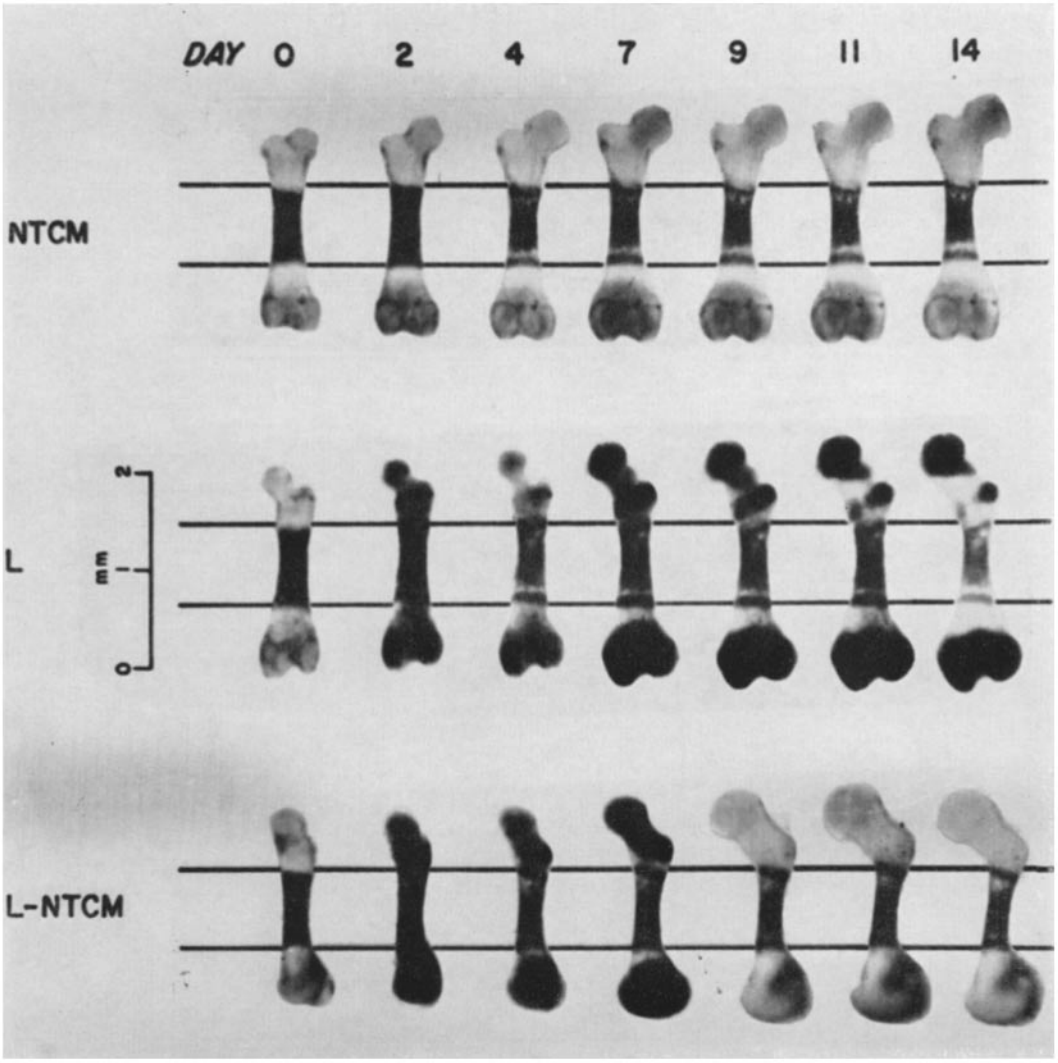


FIGURE 3 Effect of lysozyme on femur growth in vitro. Serial photographs of cultured 17-day-old embronic femurs. Note the opacification of lysozyme-treated specimens, with sparing of the cartilage-bone junction. The bottom row demonstrates the reversibility of this change. The process of reversibility is accompanied by a relative increase in the size of the explant. *NTCM* = normal tissue culture media. *L* = *NTCM* containing 1 mg/ml of lysozyme. *L-NTCM* = 7 days L, then replaced with NTCM for 7 days.

explants disappeared within 24-hr after replacement with lysozyme-free medium.

Biochemical Changes

Femurs removed from embryos and immediately inserted into hypotonic agar containing *Micrococcus lysodeikticus* induced definite rings of clearing around the tissue, which indicated that significant amounts of lysozyme were present (Fig. 2). Femurs placed into agar after 7 days in culture induced similar-sized rings of clearance, indicating that they contained amounts of lysozyme comparable to that in uncultured specimens. Uncultured dead femurs formed much smaller rings. When this assay was done on explants cultured in 0.1 or 1 mg of lysozyme/ml, the zone of clearance included almost the entire plate, rendering estimate of quantitative differences

between the samples impossible. Therefore, both viable and devitalized femurs were cultured for 24 hr in culture medium containing 10 μg of lysozyme/ml. Both types of femurs induced zones of clearance much larger than did femurs not exposed to lysozyme. Zones of clearance were larger with viable than with devitalized specimens, indicating that both living and dead embryonic femurs absorbed lysozyme from the culture medium and that the living femurs bound more lysozyme than did dead ones. It has previously been shown that the changes which lysozyme induces in cartilage in organ cultures are associated with binding of lysozyme to the tissue (16) and that these changes do not occur in cartilage which does not contain an epiphyseal growth plate (11). Thus, the presence of lysozyme in high concentrations in cartilage, and the morphological response to and binding of exogenous lysozyme appear to be interrelated properties.

Growth of the Explants

The growth of all femurs, as assessed by planimetry, was most rapid during the 1st week in culture (Fig. 3) and decreased during the 2nd week as is expected with this technique (17). Femurs cultured in medium containing 1 mg of lysozyme/ml consistently grew slightly more slowly during the 1st 2 days than their paired controls, but this difference was not significant when assayed with the *t* test. In several preliminary experiments, it was noted that when femurs maintained for 7 days in the presence of lysozyme were subsequently cultured in lysozyme-free medium, a rapid spurt of growth occurred during the following 7 days, so that these femurs became larger than the control specimens not exposed to lysozyme. This spurt of growth was most obvious in specimens from embryos obtained between the 17th and 18th day of embryonic development as judged by Wirtschafter's method (12). The changes in growth were either less obvious or absent in femurs from older or younger embryos.

A more carefully controlled experiment was then done in which femurs of embryos aged 16 or 17 days were used. Here, mouse femurs were maintained in normal tissue culture medium for 14 days; in normal tissue culture medium containing 1 mg of lysozyme/ml for 14 days; or in tissue culture medium containing 1 mg/ml of lysozyme for 7 days, and then in normal tissue culture medium without lysozyme for an additional 7 days.

The planimetric data were analyzed as follows: Separate analyses were made for each of the time intervals (i.e., 0–3 days, 3–7 days, and 7–10 days) so as to test the departure from parallelism of slopes (mean growth rates) of the three treatment groups. This was done because of the unequal time intervals between specimen area measurements (3 days and 4 days).

In the first two time intervals, the combined lysozyme groups were compared to the control group.

Observations from the prior pilot experiment suggested that after 7 days, in groups when media were unaltered, the growth rates decreased significantly. In the group grown in lysozyme for 7 days, followed by culture in a lysozyme-free medium, the data suggest that the rate of growth continued unchanged until the 10th day. Therefore, comparisons were made between the group to which exogenous lysozyme was added and then withdrawn and the other two groups combined. These comparisons were made from the data obtained between the 7th and 10th day of culture.

Because the group means were correlated with group variances, the specimen area measurements were transformed to their logarithms in order to establish homogeneity of variance essential for the validity of the analysis of variance. The analyses are shown in Table I.

Examination of the growth curves (Fig. 4) discloses differences in growth rates among the treated explants. Those explants maintained in unsupplemented medium grew in a rapid linear fashion during the 1st 7 days; this was followed by a depressed rate of growth during the 2nd wk. For the 1st 3 days, the growth rate of explants maintained in medium supplemented with lysozyme was lower than for those grown in the unsupplemented medium. The probability of identical growth rates, based on measurements in the two groups during this period, is less than 0.10, thus suggesting an initial lag phase in growth of the lysozyme-treated specimens. The slope of the growth curve from 3 to 7 days was identical with that of specimens in control cultures unexposed to lysozyme. When lysozyme was withdrawn after 7 days in culture, the slope of the curve did not change until the 10th day. The growth rates of both controls and explants con-

TABLE I

Analysis of Growth Rate Differences of 17-Day-Old Mouse Femur Explants

I. Growth rates from 0–3 days	ANOVA*			
*VS**	*DF**	*MS**	*F**	*P**
Control vs lysozyme 7 + 7 vs 14 days	1	0.00977850	1.26	NS
	1	0.14664554	18.93	<0.001
Combined slope	1	0.27843282	35.95	<0.001
Departure from parallelism				
Control vs lysozyme	1	0.01775616	2.29	0.14
7 + 7 vs 14 days	1	0.00404813	<1	NS
Within groups	40	0.00774573		
II. Growth rates from 3–7 days	**ANOVA**			
VS	*DF*	*MS*	*F*	*P*
Control vs lysozyme	1	0.04963170	5.79	<0.025
7 + 7 vs 14 days	1	0.05800143	6.77	<0.025
Combined slope	1	0.61126233	71.35	<0.001
Departure from parallelism				
Control vs lysozyme	1	0.00008764	<1	NS
7 + 7 vs 14 days	1	0.00944088	1.10	NS
Within groups	40	0.00856702		
III. Growth rates from 7–10 days	**ANOVA**			
VS	*DF*	*MS*	*F*	*P*
(Control + 14 days) vs 7 + 7	1	0.00269580	<1	NS
Control vs 14 days	1	0.00414200	<1	NS
Combined slope	1	0.14766807	13.17	<0.01
Departure from parallelism				
(Control + 14 days) vs 7 + 7	1	0.04411581	3.93	≃0.05
Control vs 14 days	1	0.00121607	<1	NS
Within groups	40	0.01121135		

* ANOVA is the standard acronym for "Analysis of Variance." *VS*, Variation source. *DF*, Degrees of freedom. *MS*, Mean square. *F*, F ratio statistic. *P*, Probability of random occurrence of difference between group means under the assumption that sampling was made from the same population.

I. 0–3 days: The analysis demonstrates significant growth in all three experimental groups in this time interval. Suggested, but not conclusive, is a slower growth rate of the two combined lysozyme groups when compared to the control group.

II. 3–7 days: The probability of (1) a mean difference of zero between the control and the two combined lysozyme groups of less than 0.025, and (2) the parallel growth rates of the two groups in this time interval reinforces the belief that lysozyme depresses the growth rate during the first 3 days.

III. 7–10 days: Significant in this time period is that termination of lysozyme in femurs previously cultured with lysozyme sustains the growth rate of the 3–7-day period, while both the control group and the group remaining in lysozyme show a significant depression of growth rate with respect to the growth rate of the previous time period.

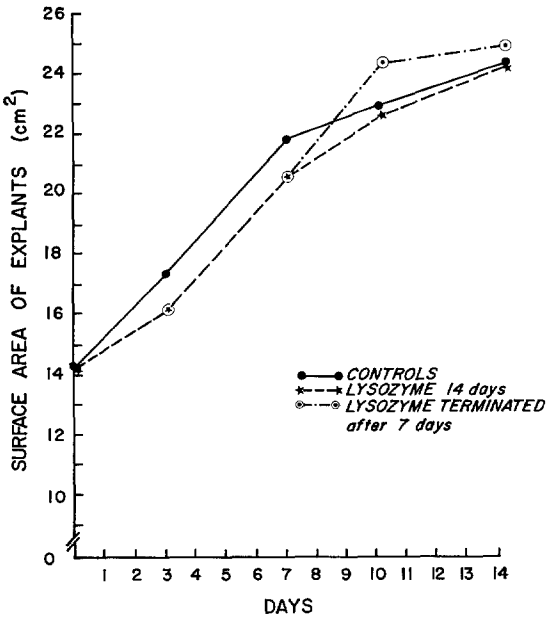


FIGURE 4 Growth rates of 17-day-old mouse femurs (Mean surface areas of explants in organ cultures 0, 3, 7, 10, and 14 days postexplantation). The slope of the curves, which indicate rate of growth of the explants, reveals an apparent repression of growth of lysozyme-containing cultures during the first 3 days in culture. Between the 3rd and 7th day, the slopes of the two groups appear to be identical. Between the 7th and 10th day, the slope of specimens maintained in lysozyme-containing medium or never exposed to lysozyme is depressed. However, for specimens which were exposed to lysozyme for 7 days and from which lysozyme was then withdrawn, the slope remains the same as during the previous 4 days. This departure from parallelism, which was statistically significant, is interpreted as a failure of growth retardation or perhaps, more descriptively, a derepression of growth.

tinuously treated with lysozyme showed a significant decrease after 7 days. Thus, in cultures which had been exposed to lysozyme for 7 days and then maintained in lysozyme-free medium, a transient but statistically significant and reproducible resumption, or perhaps more descriptively, derepression of growth occurred. This period of derepression of growth was the time in which the morphological changes, induced by lysozyme, disappeared.

It may be of importance to note that the derepression of growth observed when lysozyme was withdrawn was noted only in femurs from embryos aged 17 days, and not in those 16 or 18 days old.

DISCUSSION

These experiments were planned with the assumption that lysozyme has some role in cartilage metabolism. The evidence that the enzyme does play a role is circumstantial, but nevertheless impressive. It is present in higher concentration in preosseous cartilage than in nonossifying cartilage, and the highest concentration of it is located in areas of active proliferation and transformation into bone. Additional evidence mainly relates to variations in lysozyme content with age (10) and the recent observation that in experimental hyperparathyroidism the lysozyme content of bone, including epiphysis, decreases (18). It may not be entirely coincidental that bird egg white, which is rich in lysozyme, lies adjacent to the egg shell which, like preosseous cartilage, calcifies.

In the search for a physiological role for lysozyme, one difficulty is that, as an enzyme, lysozyme has no known substrate in mammalian tissues. This point has been extensively studied by Meyer et al. (19) who were unable to identify a substrate in cartilage which could be degraded by egg white lysozyme. Lysozyme is one of the few basic proteins which exist in tissues outside of the cell nucleus. Schubert and Franklin (20) have shown that lysozyme combines in vitro with mucoproteins and other negatively charged polyelectrolytes. The complexes formed can be easily dissociated by altering the electrolyte composition of the medium. Thus, the possibility remains that the function of lysozyme may be related to its properties as a basic protein. In this regard, it is of interest that the addition of basic proteins and polypeptides to tissue culture media increases the permeability of growing cell membranes to a variety of substances (21).

Although lysozyme in highest concentration is found in cartilage, it is also present in many other tissues. In leukocytes, lysozyme is considered to be a lysosomal enzyme (22).

The fact that lysozyme is present in leukocytes, and that it has bactericidal activity, suggests that one possible role of lysozyme may be related to the defense mechanisms of the body. If so, the response of lysozyme is not an adaptive one comparable to that of antibodies, since Wolinsky (23) has shown that the tissue concentration of lysozyme

is the same in germ-free as in conventionally bred rabbits. It seems more likely to us, however, that the antibacterial activity of lysozyme in mammalian tissues is a biological accident. This idea is, in part, based on the anatomic distribution of lysozyme in cartilage which is difficult to explain in terms of a defense mechanism. Even in other tissues, particularly in the human uterus, there is evidence that lysozyme has nonbacteriolytic activity. This evidence is, in part again, based on its anatomical distribution, since lysozyme is virtually restricted to the endocervical glands and their secretions. In addition, there is a carefully regulated response of the enzyme to the hormones of the menstrual cycle (15).

The new observations reported here can be summarized as follows: Lysozyme is present in embryonic as well as adult epiphyseal cartilage. In cultures, this cartilage can bind significant amounts of lysozyme derived from extraneous sources. In cartilage maintained in organ culture, this binding is accompanied by striking morphological changes, particularly in the matrix, and does not occur in cartilage which is not destined to become bone (11). The distribution of these changes is anatomically selective in that they affect only specific areas of the cartilage (10). Devitalized cartilage binds less lysozyme than does viable cartilage, and the morphological changes induced by lysozyme do not occur in dead cartilage. All of these changes are completely and rapidly reversible and are different from those induced by several other proteins, including protamine or lysozyme altered by UV radiation (11). During this reversal, derepression of growth occurs, but only in embryos 17 days old. This is a period of extremely rapid differentiation and bone formation in embryogenesis. We know of no clear explanation of why this effect on growth is so sharply restricted temporally during development. The morphology of the femur does not change significantly between the 16th and 18th day, and organogenesis is virtually complete at this time (25). It may be pertinent, however, that, in chick embryo femoral cartilage, lysozyme concentration varies at different periods of development, reaching a maximum on about day 16 and then declining (24).

The reason for the changes in the staining properties of the matrix of lysozyme-treated cartilage, or the significance of these changes, is not known. The changes may represent lysozyme or a reaction product between lysozyme and some other component of cartilage matrix, perhaps chondromucoprotein, which has been altered by chondrocytes and excreted into the matrix, or they may be a consequence of altered cartilage metabolism induced by lysozyme. The available information is too insufficient to permit one to choose between these alternatives at the present time, but it is known that the areas of altered staining contain lysozyme (16). The matrix of resting cartilage is more diffusely affected than that of epiphyseal cartilage. In the epiphysis, these changes selectively affect the chondrocyte lacunae and perichondrocyte matrix, not affecting the large areas of cartilage matrix between the columnar cells. The hypertrophic zone is virtually spared.

The change in growth rate upon withdrawal of lysozyme may be of particular significance not only because it suggests functional rather than merely altered staining reactions in the explanted femurs, but also because the growth spurt induced by lysozyme withdrawal is dependent upon the age of the embryo. This is particularly intriguing since it was previously shown (24) that when lysozyme was added to cultures of chick embryonic femurs, morphological effects similar to those described here occurred but only after the epiphysis had formed. Lysozyme concentration in chick embryonic cartilage varies with the age of the embryo.

The selective, anatomically distributed response of embryonic femoral cartilage to exogenous lysozyme supports the idea that lysozyme has some physiological role in this tissue. The derepression of growth which follows withdrawal of lysozyme suggests that such a function may be regulatory, particularly since it occurs only at a specific stage of embryogenesis.

The cytological location of lysozyme in cartilage is at present unknown, but in some other tissues it is lysosomal (22). If lysozyme is a lysosomal enzyme in cartilage, it must have a function somewhat different from that of other hydrolases in that organelle, since experimental procedures done in organ culture which induce release of lysosomal enzymes result in degenerative changes

in cartilage that are quite different from those induced by the addition of exogenous lysozyme to the culture medium (26–28).

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